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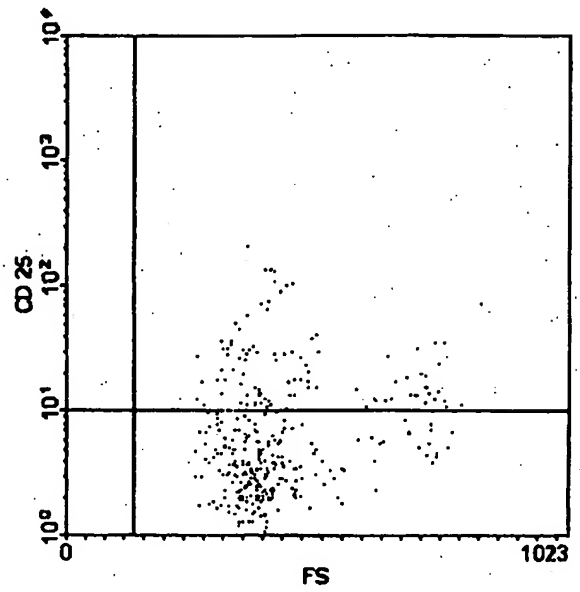
(54) Title: METHOD AND COMPOSITIONS FOR DIFFERENTIAL DETECTION OF PRIMARY TUMOR CELLS AND METASTATIC CELLS

(57) Abstract

Provided is a novel method for differentially detecting and enumerating metastatic cells present in a sample of a complex biological fluid, comprising the steps of obtaining a sample of body fluid from an individual, adding to the sample at least two detector molecules, wherein a first detector molecule has binding specificity for a cell molecule comprising IL–2Ra, and a second detector molecule has binding affinity for a cell molecule comprising an epithelial cell antigen, and the detectable moiety on the first detector molecule has a peak emission spectrum which is distinguishable from the peak emission spectrum of the detectable moiety on the second detector molecule; and analyzing cells present in the sample in a flow cytometer which is capable of detecting and distinguishing the emission spectra of the first and second detector molecules, and light scattering for each cell present in the sample. Also provided is a clinical diagnostic kit comprising a detector molecule having binding specificity for human IL–2Ra, and a detector molecule having binding specificity for an epithelial cell antigen expressed by human cells, wherein the detectable moiety on the detector molecule having binding specificity for human IL–2Ra has a peak emission spectrum which is distinguishable from the peak emission spectrum of the detectable moiety on the detector molecule having binding specificity for an epithelial cell antigen expressed by human cells. The figure is a histogram showing an epithelial cell antigen (Ber–EP4 + gataed cells (FS)) and IL–2Ra + cells (CD25).



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METHOD AND COMPOSITIONS FOR DIFFERENTIAL DETECTION OF PRIMARY TUMOR CELLS AND METASTATIC CELLS

FIELD OF THE INVENTION

The present invention relates to methods for determining if a specific subpopulation of cells is present in a sample comprising a complex biological fluid. More particularly, the present invention is directed to a flow cytometric method for the differential detection and enumeration of metastatic cells in a sample comprising a complex biological fluid that may contain an admixture of (various populations of normal and/or malignant) cells.

BACKGROUND OF THE INVENTION

Metastasis is the spread of malignant tumors to secondary sites remote from the original or primary tumor. Metastasis presents a cancer clinician with difficulty in diagnosing and treating the malignant tumor because (a) metastases may be comprised of as little as one or a few cells thereby evading clinical diagnosis even with modern techniques; (b) often metastases have already been seeded by the time a patient is diagnosed with a malignant non-lymphoid tumor (Silverberg et al., 1989, *CA Cancer J. Clin.* 39:3-21); (c) treatment is more complex than simple surgical excision of the primary tumor; (d) systemic therapy for metastatic non-lymphoid tumors, such as renal cell carcinoma (Rosenberg et al., 1985, *N. Engl. J. Med.* 313:1485-1492), remains ineffective with little survival advantage; (e) not all malignant tumors have the same metastatic potential; and (f) metastatic cells can remain dormant for long periods of time after primary tumor removal.

In general, the earlier cancer is detected, the better the chance of successful treatment and thus survival. In addition to having the potential to affect the survival of the patient, early detection of metastatic disease has other advantages. The cost of treating a tumor depends on its stage of progression, e.g., the cost of treating early

stage breast cancer may range from $10,000 to $15,000; whereas, the cost of treating advanced metastatic disease may range from $150,000 to $175,000 (Carrera, 1995, *Denver Business J.* 46:3). And in the case where a primary tumor is surgically removed, a rapid, simple and efficient method of screening for subsequent recurrence/regrowth of tumor (e.g., mediated by the spread of metastatic cells) can be a cost-effective alternative to the relatively high cost, inconvenience, and lack of sensitivity of detection of micrometastases, of post-therapeutic evaluations for residual or metastatic disease using radiographic techniques or imaging techniques (CT scans, NMR scans).

Thus, there is a need for methods to detect micrometastases. A marker for metastatic potential has been described previously. The present inventor, in U.S. Patent No. 5,536,642 (and U.S. Serial No. 08/680,372, both incorporated herein by reference), disclosed that primary tumors having a high potential to metastasize, and their metastases, express tumor cell-associated IL-2Ra. Methods are disclosed for predicting the metastatic potential of a solid non-lymphoid tumor by measuring tumor cell-associated IL-2Ra by itself, or in conjunction with a tumor cell marker associated with a desired specific tumor type. Measurement of tumor cell-associated (either internal or membrane bound) IL-2Ra in experimental tumors and human tumors shows a correlation between tumor cell-associated expression of IL-2Ra and the metastatic potential of a primary tumor, i.e. the likelihood that the primary tumor has already, or will, metastasize. While such diagnostic and prognostic methods are useful, they require a tumor specimen to measure the tumor cell-associated IL-2Ra. In that regard, problems may occur in examining tumor tissue as a specimen.

Unfortunately, for some tumor types, the primary tumor is inaccessible, or performing a biopsy of primary tumor is clinically very dangerous. Further, depending on the tumor type, concern need be exercised regarding the

possible presence of infiltrating activated T cells within the biopsy which can be a source of IL-2Ra; i.e., in T cell populations, IL-2Ra (also known as p55) is expressed on activated T cells. Additionally, often only miniscule amounts of tissue are obtained in a biopsy, thereby raising the issue of tumor sampling; i.e., the representativeness of the biopsy sample relative to the whole tumor. Lastly, the ability to detect micrometastases in complex biological fluids (e.g., the lymph and/or blood) provides a more accurate view of metastasis than does the detection of primary tumor cells having a high potential to metastasize. Detection of metastatic cells in complex biological fluids is further complicated by the expression of IL-2Ra by T lymphocytes that may normally be present in the complex biological fluid.

Hence, a need still exists for a relatively rapid, simple and efficient method for the detection and enumeration of metastatic cells in complex biological fluids. Such a method may aid in the early detection of micrometastases, whether it be micrometastases associated with tumor recurrence or regrowth, in a manner which can distinguish between metastatic cells, malignant primary tumor cells, and cells other than metastatic cells (e.g., normal blood cell populations), present in a complex biological fluid.

SUMMARY OF THE INVENTION

A primary object of the invention is to provide a method for the differential detection of metastases of solid, non-lymphoid tumors by measuring multiple parameters by flow cytometric analyses.

Another object of the invention is to provide a method for the differential detection and enumeration of metastases of solid, non-lymphoid tumors by measuring multiple parameters by flow cytometric analyses.

Another object of the invention is to provide a

method for the differential detection and enumeration of metastases of solid, non-lymphoid tumors by measuring multiple parameters by flow cytometric analysis, and which can distinguish between metastatic cells, malignant primary tumor cells, and cells other than metastatic cells (e.g., normal blood cell populations), circulating in a complex biological fluid.

A further object of the invention is to provide a method for staging malignant disease in an individual by differentially detecting and enumerating, in a complex biological fluid from the individual, metastatic cells by flow cytometric analysis using multiple parameters.

A further object of the present invention is to provide a method for the differential detection of micro-metastases in a complex biological fluid, the detection of which may be an indication of tumor recurrency or tumor regrowth

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** is a histogram showing forward scatter (FS) and side scatter (90°LS) used to gate nucleated events.

FIG. **2** is a histogram showing forward scatter (FS) and Ber-EP4 fluorescence (BER-EP4); and a new gate formed which represents Ber-EP4 positive cells ("R2").

FIG. **3** is a histogram showing Ber-EP4+ gated cells (FS) and IL-2Ra+ cells (CD 25).

FIG. **4** is a histogram illustrating the level of fluorescence background events ("Events") detected, in analysis of a negative control comprising a fluorescence-labeled IgG isotype matched antibody.

While this invention is satisfied by embodiments in many different forms, such as illustrated in the drawings herein, it is understood that the invention is described in detail as a particular embodiment of the invention so as to be considered exemplary of the principles of the invention,

and is not intended to limit the invention as determined by the appended claims and their equivalents.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

"Cell molecule" is a term used hereinafter for the purposes of the specification and claims to refer to a ligand for which a detector molecule has binding specificity, wherein the ligand is selected from the group consisting of IL-2Ra or an epithelial cell antigen. Depending on the method of preparing the sample for flow cytometric analysis, the cell molecule may either be a cell surface molecule, or an intracellular molecule.

"Complex biological fluids" or "body fluids" are terms used hereinafter for the purposes of the specification and claims to refer to body fluids of an individual in which metastatic cells and cells other than metastatic cells (normal and/or malignant) can circulate. Such fluids include, but are not limited to blood; lymph; ascitic fluid; pleural fluid; peritoneal fluid; mucous fluids (e.g., bronchial, vaginal, or prostate); other effusions associated with solid non-lymphoid tumors; and tissue cell suspensions, wherein the tissue comprises an aspirate or biopsy of liver, lung, brain, lymph node, bone marrow, adrenal gland, breast, colon, pancreas, stomach, or reproductive tract.

The term "detectable moiety" is used herein, for purposes of the specification and claims, to mean a label molecule that is directly or indirectly detectable by flow cytometry, as known to those skilled in the art of flow cytometry. The detectable moiety may be bound to a monoclonal antibody (mAb), or polyclonal antibody, or aptamer, having binding specificity for the cell molecule to be detected, using covalent or noncovalent means to form a "detector molecule" for use in a flow cytometric method. In a preferred embodiment, the detectable moiety comprises a

fluorescent molecule or fluorophore which may include, but is not limited to, fluorescein (isothiocyanate), fluorescein derivatives, phyco-erythrin, up-converting phosphors, peridinin-chlorophyll protein, fluorescamine, dansyl chloride, rhodamine, Texas red tandem, phycocyanin tandem, allo-phycocyanin tandem, and coumarin.

The term "epithelial cell antigen" is used herein, for purposes of the specification and claims, to mean a molecule found on the surface and/or in the cytoplasm of most (if not all) simple epithelial cells, but not detectable (within the limits of detection using an antibody and relative to non-specific background) with mesenchymal tissue such as lymphoid tissue or lymphocytes. Such an epithelial cell antigen may include, but is not limited to, keratin, cytokeratin, and the antigen ("Ber-EP4") recognized by mAb Ber-EP4. mAbs to keratin or cytokeratin include LE61, CAM 5.2, CK2, IT-Ks20.8, and others. Thus, an antibody with binding specificity for the epithelial cell antigen can be used with specificity to determine whether a cell reacted therewith is epithelial in nature or origin; i.e., if the cell is an epithelial cell or a tumor cell of epithelial origin or nature. Regarding the latter point, the epithelial cell antigen according to the present invention is epithelial cell-specific, and not tumor-specific (as distinguished from the tumor-specific antigens disclosed in U.S. Patent No. 5,536,642), as antibodies to epithelial cell antigen cannot distinguish between normal epithelial cells and tumor cells of epithelial origin. In contrast to the epithelial antigens which are epithelial cell-specific, there are epithelial tumor-specific antigens known to those skilled in the art, which recognize a tumor-associated antigen not normally expressed, or not accessible (e.g., cryptic) to antibody, in normal (non-neoplastic) epithelial cells of the same tissue type. Epithelial tumor-specific antigens include tumor-associated polymorphic epithelial mucin (e.g., as recognized by mAb MC5), polymorphic epi-

thelial tumor antigen, Thomas-Friedenreich-related antigen (e.g., as recognized by mAb 49H.8), and the epithelial tumor antigen recognized by mAb MoV2 (Mariani-Constantini et al., 1985, *Pathol. Res. Pract.* 180:169-80). In a preferred embodiment, the epithelial cell antigen according to the present invention is the antigen recognized by mAb Ber-EP4. mAb Ber-EP4 is directed against two glycopeptides present on the surface and in the cytoplasm of all human epithelial cells except the superficial layers of squamous epithelia, hepatocytes, and parietal cells; but does not react with mesenchymal tissue (Passlick et al., 1996, *Eur. J. of Cancer*, 32A:141-5). In addition to immunoreacting with simple epithelia, mAb Ber-EP4 has been found to immunoreact with solid non-lymphoid tumors including metastatic cells of adenocarcinoma in serous effusions(De Angelis et al., 1992, *Cytopathology* 3:111-17), ovarian carcinoma in effusions (Flynn et al., 1993, *Acta Cytol.* 37:439-47), metastatic carcinomas to the liver (Ma et al., 1993, *Am. J. Clin. Pathol.* 99:551-7). It has been reported that mAb Ber-EP4 has demonstrated a high degree (73 to 93%) of immunoreactivity with adenocarcinomas from the ovary, gastrointestinal tract, lung, and breast; and 83% immunoreactivity with malignant effusions associated therewith (Diaz-Arias et al., 1993, *Diagn. Cytopathol.* 9:516-21; Maguire et al., 1994, *Diagn. Cytopathol.* 10:130-4). Immunoreactivity of mAb Ber-EP4, and mAb AUA-1 with adenocarcinoma cells in pleural, peritoneal, and pericardial effusions was demonstrated with high sensitivity (e.g., 80% to 96%) and specificity (e.g., 81% to 100%)(Illingworth et al., 1994, *Cytopathology* 5:270-81).

The terms "metastases" or "metastatic cell" or "micrometastases" are used herein, for purposes of the specification and claims, to mean cells which have metastasized, or in the process of metastasizing, from a primary tumor wherein the primary tumor is a solid, non-lymphoid tumor, as will be more apparent from the following embodiments.

The term "monoclonal antibody" is used herein, for purposes of the specification and claims, to mean a human mAb, a murine monoclonal antibody, or engineered (e.g., recombinant) antibody molecules made therefrom which includes chimeric or "humanized" antibodies, or antibody fragments, as appreciated by those skilled in the art. The term "monoclonal antibody" also refers to a fragment of an intact antibody molecule, wherein the fragment retains all or a portion of the binding function of the whole antibody molecule; i.e., $F(ab')_2$, Fab', Fab, Fv, scFV, Fd' and Fd fragments. Methods for producing the various fragments from MAbs are well known to those skilled in the art (see, e.g., Plückthum, 1992, *Immunol. Rev.* 130:152-188). For example, $F(ab')_2$ can be produced by pepsin digestion of the monoclonal antibody, and Fab' may be produced by reducing the disulfide bridges of $F(ab')_2$ fragments. Fab fragments can be produced by papain digestion of the monoclonal antibody, whereas Fv can be prepared according to methods described in U.S. Patent No. 4,642,334. Single chain antibodies can be produced as described in U.S. Patent No. 4,946,778. Aptamers can be made against B cell determinants using methods described in U.S. Patent No. 5,789,157.

The term "parameters" is used herein, for purposes of the specification and claims, to mean indicia measurable by flow cytometers including, but not limited to, light scatter produced by a cell, electrical impedance produced by a cell, and an emission or signal produced by the detectable moiety portion of the detector molecule bound to a cell. Light scatter is the light scattered by a cell as it passes through an incident beam of light directed at the sensing region of a flow cytometer. The scattered light may be used to determine one or more cell properties, including shape, size (forward scatter), index of refraction, granularity (side scatter), and roughness. Changes in electrical impedance as each cell passes through the sensing region can be used to determine cell volume. Where a fluorescent

molecule is used as the detectable moiety of a detector molecule and the detector molecule is bound to the target cells, and as cells move (typically one by one) through a beam of excitation light from a light source (typically a laser or arc lamp), a fluorescence emission is detected at a wavelength which is typically different than the wavelength of excitation. The intensity of the fluorescence emission detected is generally proportional to the amount of the detector molecule bound to the cell; and hence can be used to determine the relative expression by that cell of the cell molecule bound, and the relative number of cells in a sample which express that cell molecule. While the type of light source used will bear on the choice of fluorescent molecules used as a detectable moiety, it should be apparent to those skilled in the art that a wide latitude of choice can be exercised in selecting one or more suitable light sources for use in the method of the present invention. For example, multiple fluorescent analyses (e.g., detecting and measuring more than one fluorescence emission from a cell having bound thereto 2 types of detector molecules with each detector molecule recognizing a different cell molecule than the other) may be performed using either a dual light source or a single light source. Regarding the single light source which essentially provides a single excitation wavelength (in view of the spectral line width), if there is sufficient spectral spacing between the emission optima of two different fluorescent molecules to permit individual detection without substantial spectral overlap, a combination of filters (and mirror) may be used to permit simultaneous detection of the spectrally separated emissions of two different, excited fluorescent molecules to provide information on multiple detector molecule use in a single flow cytometric run (see, e.g., U.S. Patent No. 4,727,020). For example, an argon laser can be used to excite a combination of a number of fluorescent molecules, the emissions of which can be spectrally separated with different filters and

photodetectors (e.g., FITC: fluorescein isothiocyanate at 525nm; PE: phycoerythrin at 575nm; PCP: peridinin-chlorophyll protein at 680nm; Texas red tandem at 610 nm, phycocyanin tandem at 640nm, and allo-phycocyanin at 660nm). Regarding a dual light source, it is known to those skilled in the art that flow cytometers useful for detecting cells can include two channels which can detect cells specifically labelled with two fluorescent molecules; and using a separate light source to excite each of the two different fluorescent molecules (see, e.g., U.S. Patent No. 4,284,412).

The term "solid non-lymphoid tumor" is used herein, for purposes of the specification and claims, to mean any primary tumor of ductal epithelial cell origin, including, but not limited to, tumors originating in the liver, lung, brain, lymph node, bone marrow, adrenal gland, breast, colon, pancreas, stomach, or reproductive tract (cervix, ovaries, endometrium etc).

The term "individual" is used herein, for purposes of the specification and claims, to mean a mammal. In a preferred embodiment, the mammal is a human.

The prognosis of a tumor bearing individual who undergoes anticancer therapy (one or more of surgery, chemotherapy, immunotherapy, and radiotherapy) is mainly determined by the extent of residual tumor load, comprising either primary tumor and/or presence of micrometastases (occult to current imaging techniques), following anticancer therapy. Thus, a method for the detection of primary tumor cells and metastatic tumor cells, and for differentially detecting and enumerating primary tumor cells and their metastases, may provide information clinically significant to the tumor bearing individual. The present invention is directed to a flow cytometric method for the identification and enumeration of metastatic cells in a sample comprising a complex biological fluid that may contain an admixture of

(various populations of normal and/or malignant) cells. In a preferred embodiment, differentially detected are primary tumor cells and/or micrometastases of solid non-lymphoid tumors in blood or other body fluids of an individual. In that regard, measured in a complex biological fluid are cells expressing either epithelial cell antigen (representing a primary tumor cell) or a combination of epithelial antigen and IL-2Ra (representing a metastatic cell) in conjunction with other parameters by flow cytometry. In a preferred embodiment, the epithelial cell antigen is Ber-EP4. Using the method according to the present invention, the detection in complex biological fluid of cells expressing an epithelial cell antigen, or a combination of epithelial cell antigen and IL-2Ra, can be used for aiding in the diagnosis of metastases (an accurate assessment of micrometastases present in a complex biological fluid); providing an accurate assessment of residual cancer; monitoring an individual in cancer remission for recurrence; early detection of cancer recurrences; and staging the malignant disease in a tumor bearing individual.

In the method according to the present invention, a body fluid is obtained. A sample body fluid is analyzed according to the method of the present invention using multiple parameters by flow cytometry. The multiple parameters comprise at least two measures of light scatter for each cell examined; and at least two measures of fluorescence emission or activity for each cell examined, wherein each fluorescence emission is associated with a detector molecule having specificity for a cell molecule, and wherein the cell molecules comprise an epithelial cell antigen and IL-2Ra. The body fluid sample is combined with at least two detector molecules, each detector molecule differing in the cell molecule for which they have binding specificity, and differing in detectable moiety bound thereto. Since each detectable moiety has a peak emission spectrum that is distinguishable from the others, and

because the cell molecules are differentially expressed on cells of specific subpopulations in a body fluid, the method of the present invention may differentially detect tumor cells, and metastatic cells, in an admixture of cells. The sample is then introduced into a flow cytometer, wherein each cell in the sample is examined substantially one at a time, and measurements of the emission spectra and light scatter are measured for each cell. The measurements taken for each cell examined may then be stored in a system for data storage and analysis to allow for manipulation and generation of data.

The method of the present invention is a method for discriminating between primary tumor cells (including primary tumor cells residual from anticancer treatment), metastatic cells, and nonmalignant cells that may be present in a sample of body fluid. Desirably, the method of the present invention comprises the steps of: obtaining a sample of body fluid from an individual; adding to the sample at least two detector molecules, wherein a first detector molecule has binding specificity for a cell molecule comprising IL-2Ra, and a second detector molecule has binding specificity for a cell molecule comprising an epithelial cell antigen, and the detectable moiety on the first detector molecule has a peak emission spectrum which is distinguishable from the peak emission spectrum of the detectable moiety on the second detector molecule; and analyzing cells present in the sample in a flow cytometer which is capable of detecting and distinguishing the emission spectra of the detector molecules, and light scattering for each cell present in the sample; wherein primary tumor cells (including residual tumor) present in the sample are detectable by expression of the epithelial cell antigen, and the metastatic cells are detectable by the expression of both the epithelial cell antigen and IL-2Ra. Thus, using the method according to the present invention, primary tumor cells are differentially distinguished from

metastatic cells present in a body fluid sample. It will be appreciated by those skilled in the art that analysis of the sample may be performed in two separate procedures by splitting the sample, and analyzing one sample for cells expressing an epithelial cell antigen, and cells expressing both human IL-2Ra and epithelial cell antigen, with the appropriate gating for nucleated cells. Also provided with the present invention is an assay kit for detecting primary tumor cells, metastatic cells, or a combination thereof, in a body fluid; wherein the kit comprises a detector molecule having binding specificity for human IL-2Ra, a detector molecule having binding specificity for an epithelial cell antigen expressed by human cells, wherein the detectable moiety on the detector molecule having binding specificity for human IL-2Ra has a peak emission spectrum which is distinguishable from the peak emission spectrum of the detectable moiety on the detector molecule having binding specificity for an epithelial cell antigen expressed by human cells. The kit can further comprise a reagent selected from the group consisting of positive controls, a negative control, buffers for the incubation of the detector molecules with the sample, a fixative for cells present in the sample, a reagent for lysing red blood cells, and a combination thereof.

EXAMPLE 1

This example illustrates one embodiment of the method according to the present invention for discriminating between primary tumor cells (including primary tumor cells residual from anticancer treatment), metastatic cells, and nonmalignant cells that may be present in a sample of body fluid. A number of commercially available flow cytometers can be used as the instrument on which is performed the method of the present invention. Desirably, the flow cytometer has a single laser source; and in a preferred

embodiment, the single laser source is an argon laser tuned at 488 nanometers (nm). Additionally, the flow cytometer is operatively connected to appropriate operating software and data management systems.

A sample of the body fluid to be analyzed can be processed using anyone of several methods known to those skilled in the art. If the body fluid is peripheral blood, the sample may, but it is not required to, be treated to remove erythrocytes by centrifugation, or with a lysing agent to lyse erythrocytes present in the sample. Such a lysing agent includes 1% NH_4Cl or other commercially available lysing solutions (for a review, see Tiirikainen, 1995, *Cytometry*, 20:341-8). Similarly, if a tissue aspirate or effusion was significantly contaminated by blood during collection, erythrocytes may be removed prior to loading the sample to be analyzed onto the flow cytometer. Alternatively, a large range of light scatter signals is obtained using unlysed samples. Since platelets, erythrocytes and cell debris are typically less than 8 microns (m), and primary tumor cells and metastatic cells are typically 8-20m or larger, forward and side light scatter signals can be gated for size and/or to nucleated events (e.g. to exclude platelets, erythrocytes and cell debris from subsequent analysis). In that regard, nucleated events were gated by setting the instrumental threshold to exclude smaller cells and debris.

The sample may be prepared for analysis using any one of a number of methods for sample preparation for flow cytometric analysis known to those skilled in the art. For example, the sample may be adjusted for cell density by diluting the sample in a physiological buffer (e.g., phosphate buffered saline (PBS); PBS with 1% bovine serum albumin). The sample, preferably containing 100,000 to 200,000 cells in a small volume (e.g., ranging from 20ml to 100ml), was incubated with at least two different pretitered detector molecules for 20-30 minutes at 4°C. After this

incubation, optional further washes may be made in physiological buffer. The sample may be further diluted to a final volume for analysis on the flow cytometer. The cells in the sample do not need to be fixed. However, if desired, cells in the sample may be fixed prior to staining with detector molecules and may be permeabilized by adding a non-ionic detergent (for detecting cell molecules in the cytoplasm and on the cell surface), or after staining with detector molecules (for detecting cell surface molecules). Cells can be fixed by incubating them with any one of a number of solutions known in the art to include, but are not limited to, 1% paraformaldehyde, methanol, methanol/acetone, acetone, 2% (v/v)paraformaldehyde and acetone, and 70% ethanol.

In one illustration of the method according to the present invention, cells in the sample were immunostained with a first detector molecule comprised PE-conjugated mAb against IL-2Ra for detecting cell molecule IL-2Ra; and a second detector molecule comprised FITC-conjugated mAb against Ber-EP4 for detecting cell molecule epithelial cell antigen. As negative controls, an aliquot of the sample can be incubated with the appropriate detectable moiety labelled IgG isotype controls. The sample is then analyzed on a flow cytometer by exciting the fluorescence using an argon laser at 488 nm. The fluorescent signals were collected through a 530 nm band pass filter for the FITC emissions, and a 585 nm band pass filter for the PE emissions.

In a first parameter of analysis, forward scattering and side scattering are measured. As illustrated in the histogram depicted in FIG. 1, forward scatter (FS) and side scatter (90°LS) may be used to gate nucleated events (i.e., select for the size and granularity representative of nucleated cells). As illustrated in the histogram depicted in FIG. 2, and in the correlation between forward scatter (FS) and Ber-EP4 fluorescence (BER-EP4), a new gate is formed which represents Ber-EP4 positive (+) cells ("R2",

representing all gated nucleated cells of epithelial origin). The Ber-EP4+ cells are then correlated with IL-2Ra+ cells to detect cells positive for both Ber-EP4 (FS) and IL-2Ra (CD 25) as shown in the histogram depicted in FIG. 3. To enumerate the number of Ber-EP4+/IL-2Ra+ cells (cells expressing both Ber-EP4 and IL-2Ra), a threshold level should be set in relation to a negative control. The histogram depicted in FIG. 4 illustrates that the level of fluorescence background events ("Events") detected, in analysis of a negative control comprising a PE-labelled IgG isotype matched antibody, is 10^1 (note width of marker bar in FIG. 4) Thus, using 10^1 as a threshold, a quadrant was applied to the histogram depicted in FIG. 3 to provide for accurate enumeration of Ber-EP4+/IL-2Ra+ cells. The events present in the upper right quadrant of FIG. 3 represent the Ber-EP4+/IL-2Ra+ cells. Using these or similar parameters, gatings, and thresholds allows for a small margin of error. In this illustration (FIGs. 1-4), the margin of error was 0.03%. Using the flow cytometer statistical analysis software, the number of metastatic cells present in the sample (Ber-EP4+/IL-2Ra+ cells) can be determined relative to the primary tumor cells in the sample (Ber-EP4+ cells). In this illustration, 30.54% of the tumor cells were metastatic cells. Such a high percentage of metastatic cells found in a sample from an individual is highly indicative that metastasis has occurred in this individual.

EXAMPLE 2

Using the method according to the present invention, normal tissue (lymph node) biopsy aspirates were obtained from several patients not bearing clinically evident cancer. The samples were analyzed for staining with detector molecules to detect Ber-EP4 and IL-2Ra cell expression. The preparation, staining, and flow cytometry set-up procedures were performed as essentially described in Example 1. As shown in Table 1, of the 6 lymph nodes ana-

lyzed, no significant (99% confidence level) staining with Ber-EP4 was detected. This supports the reported selective expression of Ber-EP4 as being cells of epithelial origin, while excluding all lymphatic cells, endothelial cells, and hematopoietic cells.

Table 1

Lymph Node Specimen	% Ber-EP4 +
1	0.02
2	0.00
3	0.00
4	0.01
5	0.01
Average	**0.008**

Additionally, samples of tissue biopsy aspirates were obtained from thirteen women ranging in ages from 39 years old to 63 years old with a clinical history of breast cancer. Neoplastic disease included infiltrating ductal carcinoma, extensive ductal carcinoma, and intraductal and infiltrating carcinoma ("CA", Table 2). Also, tissue biopsy aspirates were obtained from lymph nodes regional to a carcinoma ("LN", Table 2). The preparation, staining, and flow cytometry set-up procedures were performed as described in Example 1. Some of the samples had also been analyzed by histochemical staining using hematoxylin and eosin, and visualization by light microscopy. Table 2 shows the percentage of primary tumor cells present in the sample (Ber-EP4+), the percentage of metastatic cells present in the sample (Ber-EP4+/IL-2Ra +) obtained by the method according to the present invention. Additionally, where the data was available, the results obtained by the method according to the present invention were compared to the results obtained by histochemical staining (H&E) of lymph node samples (number of positive lymph nodes/total number of

lymph nodes analyzed) from the relevant patient.

Table 2

Patient/Sample		%Ber-EP4 +	%Ber-EP4+/IL-2Ra +	H&E: #LN+/LN
1	CA	0	0	-
2	CA	0	0	-
3	LN	2.3	0.19	-
4	CA	10.28	0.45	-
5	CA	0.47	2.11	-
6	CA	5.82	9.91	-
7	CA	1.56	0	-
8	LN	0.25	19.14	4/19
9	CA	10.98	0.25	6/30
10	CA	3.77	8.23	24/26
11	CA	21.1	1.55	3/41
12	CA	11.47	2.01	-
13	CA	15.0	1.79	-

EXAMPLE 3

Using the method according to the present invention, additional samples of tissue biopsy aspirates were analyzed for differentially detecting and enumerating metastasizing cells. The preparation, staining, and flow cytometry set-up procedures were performed as described in Example 1.

Case #1: Previous cancer was a grade I melanoma, with no recurrence reported. Six years later, an adenopathy raised concern for recurrence. The pathology report indicated that it was a benign process with no tumor involvement. A sample of a lymph node aspirate which was analyzed according to the present invention showed 0.00% Ber-EP4+/IL-2Ra + cells. This is consistent with selective expression of Ber-EP4 as being cells of epithelial origin, and not melanoma cells.

Case #2: Previously reported was a breast carcinoma, with recurrence reported. Thirty axillary lymph nodes were obtained in surgery; and the pathology report indicated that six contained metastases. A sample of a lymph node aspirate which was analyzed according to the present invention showed 4.7% Ber-EP4+/IL-2Ra + cells.

Case #3: Reported was a poorly differentiated breast carcinoma. Twenty axillary lymph nodes were obtained in surgery; and the pathology report indicated metastasis. A sample of a tumor aspirate which was analyzed according to the present invention showed 18.0% Ber-EP4+/IL-2Ra + cells. Samples of two different lymph node aspirates which were analyzed according to the present invention showed 3.1% Ber-EP4+/IL-2Ra + cells and showed 10.2% Ber-EP4+/IL-2Ra + cells, respectively.

Case #4: Previously reported was a intraductal breast carcinoma, with recurrence reported. Tumor biopsy was obtained in surgery; and the pathology report indicated metastasis. A sample of an biopsy aspirate which was analyzed according to the present invention showed 48.6% Ber-EP4+/IL-2Ra + cells.

Case #5: Reported was an intraductal breast carcinoma. Thirty three axillary lymph nodes were obtained in surgery; and the pathology report indicated no metastasis. A sample of a tumor aspirate which was analyzed according to the present invention showed 19.0% Ber-EP4+/IL-2Ra + cells. A sample of a lymph node aspirate which was analyzed according to the present invention showed 2.9% Ber-EP4+/IL-2Ra + cells.

Case #6: Reported was intraductal breast carcinoma. Twenty six axillary lymph nodes were obtained in surgery; and the pathology report indicated metastasis in twenty four of the

nodes. A sample of a single lymph node aspirate which was analyzed according to the present invention showed 12.0% Ber-EP4+/IL-2Ra + cells.

Case #7: Reported was intraductal breast carcinoma. Nineteen axillary lymph nodes were obtained in surgery; and the pathology report indicated metastasis in four of the nodes. A sample of a the biopsy aspirate which was analyzed according to the present invention showed 2.9% Ber-EP4+/IL-2Ra + cells.

Case #8: Reported was intraductal breast carcinoma. Forty one axillary lymph nodes were obtained in surgery; and the pathology report indicated no metastasis. A sample of the biopsy aspirate which was analyzed according to the present invention showed 2.9% Ber-EP4+/IL-2Ra + cells.

Case #9: Reported was intraductal breast carcinoma. Several axillary lymph nodes were obtained in surgery; and the pathology report indicated no metastasis. A sample of the biopsy aspirate which was analyzed according to the present invention showed 1.8% Ber-EP4+/IL-2Ra + cells.

Case #10: Reported was intraductal breast carcinoma. Several axillary lymph nodes were obtained in surgery; and the pathology report indicated no metastasis. A sample of the biopsy aspirate which was analyzed according to the present invention showed 4.7% Ber-EP4+/IL-2Ra + cells.

EXAMPLE 4

The method according to the present invention provides a more authentic view of tumor progression, in differentially detecting and enumerating metastatic cells in a body fluid, than procedures currently utilized clinically. Current methods of disease staging often fail to detect micrometastases in body fluids. Metastatic relapse may arise

from such micrometastases. Concomitant use of the epithelial cell antigen and IL-2Ra as markers, may be useful in establishing a sensitive and statistically appropriate alternative standard for evaluating progression or recurrence of tumor in patients. For example, because of the problems that metastases present, in terms of diagnosis and treatment of non-lymphoid tumors, a method for differentially detecting metastatic cells is desirable. As described above, detection of both epithelial cell antigen and IL-2Ra markers correlates with the percentage or number of metastatic cells present in a sample, and thus may be used for determining the staging of tumor progression, and the patient outcome (i.e., a large number of cells in a sample which stain positive for both epithelial cell antigen and IL2Ra is associated with a poorer prognosis) and/or may be an indicator that metastatic recurrent has occurred.

As described above, the double marker system of the present invention comprises analyzing a sample of cells for epithelial cell antigen and IL-2Ra expression. Detection of cells expressing both markers correlates with the number of metastatic cells in a body fluid of a patient; and therefore may provide an accurate indicator of the progression of malignant disease in that patient. A method for determining the stage of malignant disease may involve periodic measurements for cells expressing both epithelial cell antigen and IL-2Ra levels in the appropriate body fluid(s) (i.e., depending on the tumor tissue type) using a clinical diagnostic kit for determining expression by a cell of both epithelial cell antigen and IL-2Ra. The number of cells detected in each measurement are then compared to determine if there is an increase (an indicator of advancing stages, and a poorer prognosis), a decrease (an indicator of a decrease in malignant disease), or a constant level (maintaining the same stage of malignant disease). A method for determining the staging of tumor progression (e.g., by detecting and enumerating metastatic cells in a sample)

comprises measuring the cell expression of both epithelial cell antigen and IL-2Ra in the appropriate body fluid(s) (i.e., depending on the tumor tissue type) using a clinical diagnostic kit for determining epithelial cell antigen and IL-2Ra expression. Such a clinical diagnostic kit may comprise, for example a detector molecule having binding specificity for human IL-2Ra, a detector molecule having binding specificity for an epithelial cell antigen expressed by human cells, wherein the detectable moiety on the detector molecule having binding specificity for human IL-2Ra has a peak emission spectrum which is distinguishable from the peak emission spectrum of the detectable moiety on the detector molecule having binding specificity for an epithelial cell antigen expressed by human cells. The kit may further comprise a reagent selected from the group consisting of positive controls (e.g., a detector molecule such as for a tumor specific antigen), a negative control (antibodies or detector molecules which are isotype matched with the detector molecules for epithelial cell antigen and IL-2Ra), buffers for the incubation of the detector molecules with the sample and for flow cytometric analysis, a fixative for cells to be analyzed, a reagent for lysing red blood cells, and a combination thereof.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, various modifications will become apparent to those skilled in the related arts from the foregoing description and figures. For example, analysis of cells expressing both epithelial cell antigen and IL-2Ra may be performed manually by mounting the sample and analyzing the stained sample by fluorescence microscopy. Such modifications are intended to be included within the spirit of this application and within the scope of the appended claims.

What is claimed is:

1. A method for differentially detecting metastatic cells that may be present in a sample of body fluid comprising the steps of:

(a) obtaining a cell-containing sample of body fluid from an individual;

(b) adding to the cells at least two detector molecules, wherein a first detector molecule has binding specificity for a cell molecule comprising IL-2Ra, and a second detector molecule has binding specificity for a cell molecule comprising an epithelial cell antigen, and the detectable moiety on the first detector molecule has a peak emission spectrum which is distinguishable from the peak emission spectrum of the detectable moiety on the second detector molecule;

(c) analyzing cells present in the sample in an instrument which is capable of detecting and distinguishing the emission spectrum of one or more of the detector molecules when bound to the cells, and capable of detecting the size and granularity for each cell present in the sample;

wherein metastatic cells are detected by the expression of both the epithelial cell antigen and IL-2Ra.

2. The method of claim 1 wherein the detectable moieties are selected from the group consisting of fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll protein, Texas red tandem, phycocyanin tandem, and allo-phycocyanin.

3. The method according to claim 1, wherein the size and granularity of each cell is detected by measuring forward light scatter and side scatter.

4. The method of claim 3, wherein the measured forward light scatter and side scatter are used to gate nucleated events.

5. The method of claim 1, wherein erythrocytes are removed from the sample prior to analysis, and wherein the erythrocytes are removed by a process selected from the group consisting of centrifugation, and lysis by the addition of a lysing reagent.

6. The method of claim 1, wherein the cells in the sample are fixed prior to analysis by adding a fixative to the sample.

7. A method for differentially detecting and enumerating metastatic cells that may be present in a sample of body fluid comprising the steps of:

(a) obtaining a cell-containing sample of body fluid from an individual;

(b) adding to the cells at least two detector molecules, wherein a first detector molecule has binding specificity for a cell molecule comprising IL-2Ra, and a second detector molecule has binding specificity for a cell molecule comprising an epithelial cell antigen, and the detectable moiety on the first detector molecule has a peak emission spectrum which is distinguishable from the peak emission spectrum of the detectable moiety on the second detector molecule;

(c) analyzing cells present in the sample in an instrument which is capable of detecting and distinguishing the emission spectrum of one or more of the detector molecules when bound to the cells, and capable of detecting the size and granularity for each cell present in the sample;

(d) detecting metastatic cells present in the sample by determining the number of cells in the sample expressing both the epithelial cell antigen and IL-2Ra;

(e) detecting tumor cells present in the sample by determining the number of cells in the sample expressing epithelial cell antigen; and

(f) enumerating the metastatic cells present in the sample by calculating the percentage of metastatic cells relative to nucleated cells present in the sample.

8. The method of claim 7 wherein the detectable moieties are selected from the group consisting of fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll protein, Texas red tandem, phycocyanin tandem, and allo-phycocyanin.

9. The method according to claim 7, wherein the size and granularity of each cell is detected by measuring forward light scatter and side scatter.

10. The method of claim 9, wherein the measured forward light scatter and side scatter are used to gate nucleated events.

11. The method of claim 7, wherein erythrocytes are removed from the sample prior to analysis, and wherein the erythrocytes are removed by a process selected from the group consisting of centrifugation, and lysis by the addition of a lysing reagent.

12. The method of claim 7, wherein the cells in the sample are fixed prior to analysis by adding a fixative to the sample.

13. A clinical diagnostic kit for differentially detecting metastatic cells comprising a detector molecule having binding specificity for human IL-2Ra, a detector molecule having binding specificity for an epithelial cell antigen expressed by human cells, wherein a detectable moiety on the detector molecule having binding specificity for IL-2Ra has a peak emission spectrum which is distinguishable from the peak emission spectrum of a detectable moiety on the

detector molecule having binding specificity for an epithelial cell antigen.

14. The kit according to claim 13, wherein the kit further comprises a reagent selected from the group consisting of a positive control, a negative control, buffers for incubating of the detector molecules with a sample and for flow cytometric analysis, a fixative for cells to be analyzed, a reagent for lysing red blood cells, and a combination thereof.

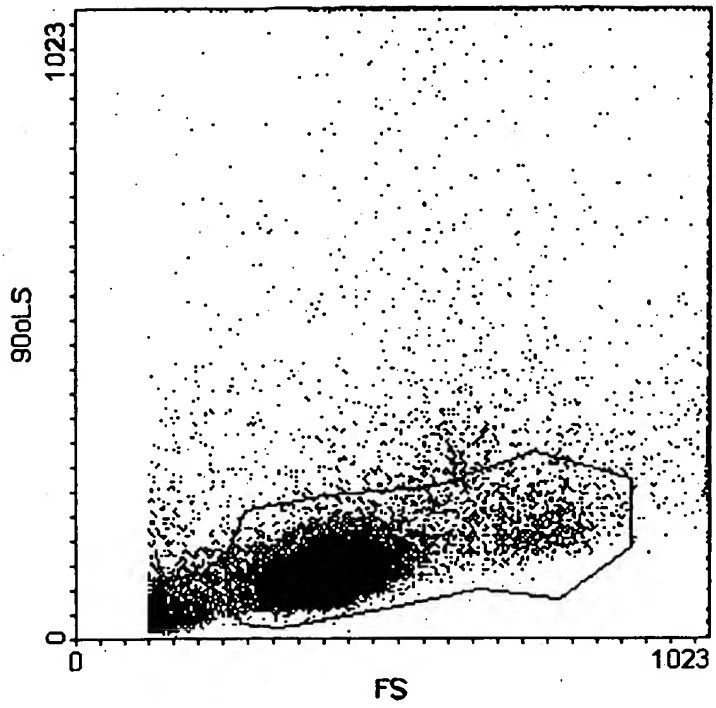


FIG. 1

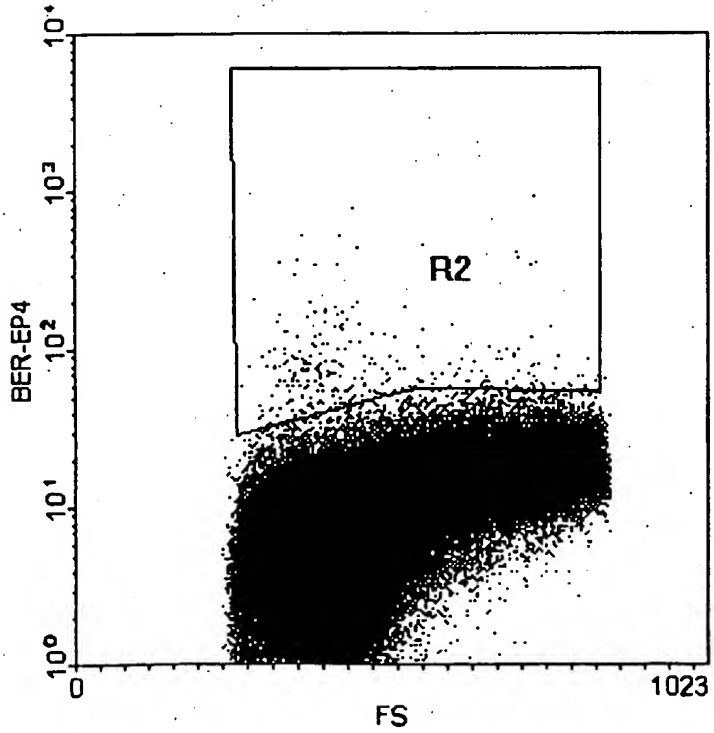
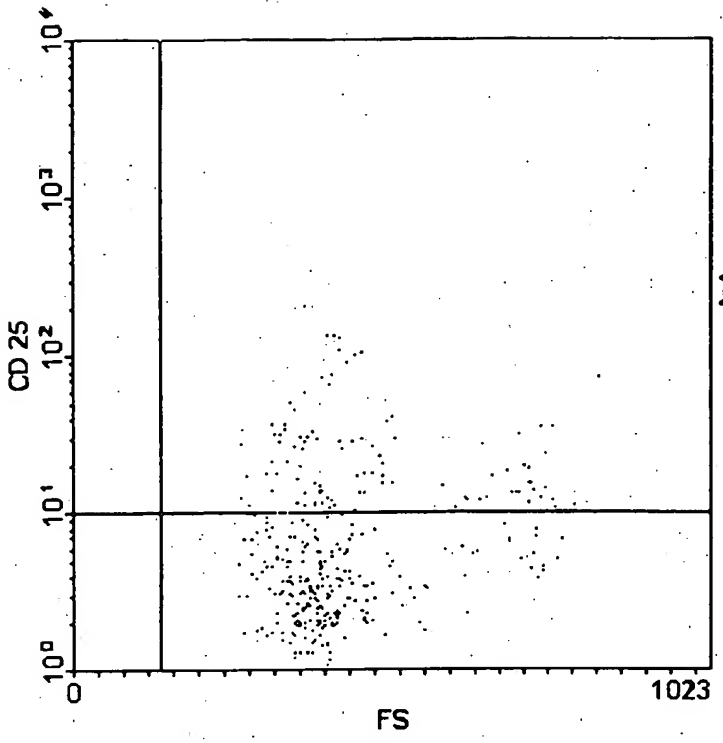


FIG. 2


CD 25
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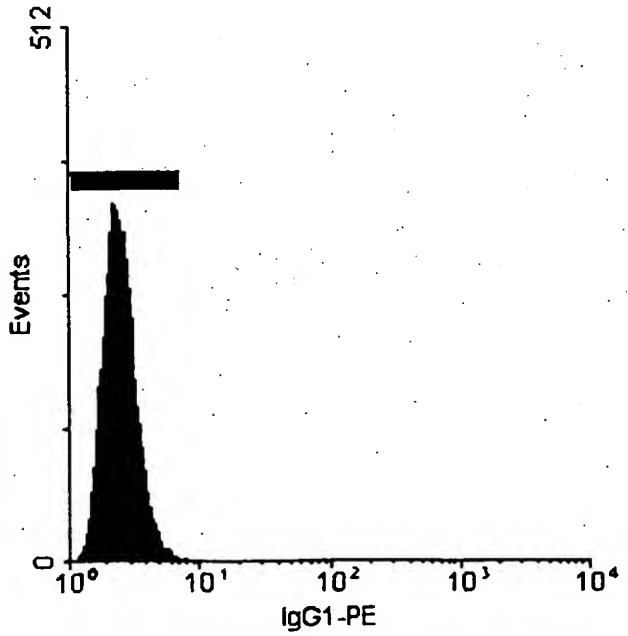
10^3
10^2
10^1
10^0
0
1023
FS

FIG. 3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10^2
10^3
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IgG1-PE

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04005

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :G01N 33/53
US CL :435/7.23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 4,6,7.21,7.24,7.9,34,64,85.2,813; 436/63,72,94,501,519,548,800,805,827; 424/12, 356/36.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN
serach terms: IL-2R.alpha, Ber-EP4, metastases, tumor, cancer, carcinoma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,536,642 A (BARBERA-GUILLEM et al.) 16 July 1996. See entire document.	1-14
Y	US 4,284,412 A (HANSEN et al.) 18 August 1981. Abstract, Summary, col. 5, lines 40-57.	1, 3-5, 7-11
Y	US 4,727,020 A (RECKTENWALD) 23 February 1988. See Abstract, column 1, lines 47-68, col. 2, lines 45-49, Summary, col. 4, lines 20-64, col. 5 and 6.	1--5, 7-10
A	US 5,569,585 A (GOODWIN et al.) 29 October 1996. See entire document.	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 JUNE 1999	Date of mailing of the international search report 01 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GAILENE R. GABEL JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04005

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BARTON et al. Expression of Interleukin-2 Receptor Alpha (IL-2R.α) mRNA and Protein in Advanced Epithelial Ovarian Cancer. Anticancer Research. 1994, Vol. 14 pages 761-772, see entire document.	1-14